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Discovery of two Palearctic *Bootanomyia* Girault (Hymenoptera, Megastigmidae) parasitic wasp species introduced to North America

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Abstract

Invasive species are among the greatest threats to ecosystems, but our ability to detect species introductions varies across taxa. Parasitoid wasps, though one of the most species-rich groups of all animals, are small and have ephemeral adult stages, such that they often go unnoticed. Here, we document two separate introductions of European parasitoids of oak gall wasps into North America. Both wasps key morphologically to *Bootanomyia dorsalis* (Fabricus), which previous genetic data from Europe suggest comprises two distinct species, *B. dorsalis* sp. 1 and *B. dorsalis* sp. 2. We find *B. dorsalis* sp. 1 in oak galls from New York, USA and *B. dorsalis* sp. 2 in oak galls from Washington, Oregon, and British Columbia, Canada. All oak gall wasp hosts were North American natives. We detect no genetic variation at the mtCOI locus within *B. dorsalis* sp. 2 specimens, suggesting this introduction may have had only a small number of founder individuals. In their native ranges, both species attack several different gall wasp hosts, and we likewise reared both from galls of multiple North American gall wasp hosts, suggesting a potential for widespread impact on North American gall insect communities. These introductions were detected only because our research groups are actively sampling and identifying parasitoid communities across gall habitats. Most parasitoid communities are not regularly sampled across hosts, time and space, or are well characterized, such that many more undetected wasp introductions may be impacting native insects worldwide.

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Keywords
Cynipidae, gall wasp, Nearctic, non-native, parasitic wasp, *Quercus*

Introduction

The ability to detect introduced and potentially invasive species varies greatly across different types of organisms (Crystal-Ornelas and Lockwood 2020). Insects represent the most frequently introduced group of animals to non-native ranges (Sodhi and Ehrlich 2010; Seebens et al. 2017; Pagad et al. 2022). One category of insects, the parasitoid wasps, may be the most species-rich of all animals (Forbes et al. 2018), but are not commonly cited invaders and often go undescribed even in their native ranges, due to their small size and ephemeral adult stages (Saunders and Ward 2018). This combination of high species richness with challenging detection suggests there could be extensive undocumented parasitoid wasp introductions. This potential for under-detection is a critical problem given the important role of parasitoids in regulating the population sizes of other insects (Gómez and Zamora 1994).

One low-tech but high-confidence approach to detection of parasitic wasps is to collect potential insect hosts and then rear and identify any wasps that emerge. Our research team collects and rears insects from galls induced on oaks by wasps (Cynipidae: Cynipini) in North America to better understand their diversity and evolution. Oak gall wasps lay eggs into the meristematic tissue of oak trees and induce the tree to form galls—growths of plant tissue that provide food and putative shelter to the developing wasp larvae (Egan et al. 2018). This work has resulted in new species discovery (Ward et al. 2020; Sheikh et al. 2022), species descriptions (Egan et al. 2017; Nastasi et al. 2024), evolutionary patterns (Ward et al. 2020; Ward et al. 2022b; Ward et al. 2024), and patterns in diversity and interactions (Prior et al. 2023). These investigations have also revealed extensive, and previously undocumented, parasitic wasp diversity associated with oak galls (Ward et al. 2022a).

Some surprising associates of cynipid oak galls among our collections have been wasps from the genus *Bootanomyia* Girault (Hymenoptera: Megastigmidae). Species in this genus are common parasites of cynipid gall forming wasps on oaks in the Palearctic (Doğanlar, 2011), but there were no published records of Nearctic *Bootanomyia* associated with oak galls prior to our surveys. In two recent papers, we noted the presence of wasps fitting the description of the Palearctic species *Bootanomyia dorsalis* (Fabricus) reared from four different host galls native to the Pacific coast of North America (Ward et al. 2022a; Prior et al. 2023). We considered the possibility that these might represent an introduction (Ward et al. 2022a; Jones 2023) but did not test that hypothesis. Separately in 2023 and 2024, we reared *Bootanomyia* wasps from native North American oak galls collected at the Stone Barns Center for Food and Agriculture in Westchester Co., NY and near the Brooklyn Bridge Park Conservancy in Brooklyn Co., NY. These new collections, more than three thousand kilometers from the Pacific coast wasps, inspired us to test whether North American *Bootanomyia* from oak galls represent introductions of Palearctic species, or if they instead could be previously undetected species native to the Nearctic.

Methods

Collections and rearing

We collected Pacific coast (northern CA, OR, WA, and southwest BC) galls exclusively from Oregon White Oak (*Quercus garryana* Dougl. Ex Hook.), a white oak (section Quercus). These collections focused primarily on 23 gall morphotypes (Jones et al. 2022; Prior et al. 2023). Collections from the east coast targeted more than 30 oak species from both sections Quercus and Lobatae (red oaks) and included more than 200 gall morphotypes (Ward et al. 2022a; Brown et al. unpublished data). All gall wasp and oak tree species were native to North America. We identified galls to morphotype and placed them into containers for rearing in either environmental chambers or under ambient conditions (Westchester Co.). We checked rearing containers once per week for the Pacific collections and daily for the eastern collections for emerging insects. We immediately placed all adult insects into tubes of 95% EtOH. For more collection and rearing details see Jones et al. (2022), Prior et al. (2023), and Ward et al. (2022a). We identified all *Bootanomyia* specimens morphologically using a key in Doğanlar (2011). We photographed representative specimens using a Leica S9i digital stereo microscope (Leica Microsystems, Heerbrugg, Switzerland) with an integrated 10-megapixel camera at 5x magnification. Some point mounted images were stacked using Zerene Stacker (Zerene Systems, LLC, Richland, WA).

Genetic identification

We sequenced 19 Pacific coast *Bootanomyia* specimens and four NY specimens. West coast samples were destructively extracted using a Qiagen PureGene standard protocol for DNA purification from tissue, excluding the RNase A step. One New York specimen was also destructively extracted, while for the second (Westchester Co.) we removed the legs from one side of the body for destructive extraction and then used a non-destructive method on the remaining tissue. We extracted DNA from both New York samples using a modified CTAB protocol (Chen et al. 2010). Two New York specimens and two Pacific coast specimens were pinned and submitted to the University of Iowa Museum of Natural History (SUI_INS 39342–39345). We PCR-amplified the mitochondrial locus Cytochrome Oxidase Subunit I (mtCOI) using primers for pF2 (5'-ACC WGT AAT RAT AGG DGG DTT TGG DAA-3') and 2437d (5'-GCT ART CAT CTA AAW AYT TTA ATW CCW G-3') (Simon et al. 1994; Kaartinen et al. 2010). The conditions for PCR were an initial denaturation at 95 °C for 1 min followed by forty repeated cycles of denaturation at 95 °C for 30 sec, annealing at 48 °C for 30 sec, and elongation at 72 °C for 30 sec. After these cycles, we performed a final elongation at 72 °C for 5 min and recovered at 4 °C. Amplicons from the Pacific coast samples were sequenced using Sanger Sequencing by Eurofins (Louisville, KY), and reads were processed in Geneious version 2024.0.5 (Biomatters, LLC, New York, NY) for final consensus sequences. For PCR on the NY specimens, primers were tagged with distinct 13 bp oligonucleotide barcodes designed by Srivathsan et al. (2021) such that each amplicon was distinguishable from others. Primer sequences and their barcodes can be found in Suppl. material 1: table S1. Amplicons were combined

into a single library and prepared for sequencing on an Oxford Nanopore MinION Mk1B platform with a V14 (SQK-LSK114) Ligation Sequencing Kit (Wang et al. 2021). We sequenced the library using a R10.3 chemistry Flongle Flow Cell. We demultiplexed reads using a custom Snakemake workflow (Mölder et al. 2021) that implemented the following steps: demultiplexing using minibar v0.24 (Calacademy-Research/Minibar: Dual Barcode and Primer Demultiplexing for MinION Sequenced Reads) then dereplication, clustering, and chimera removal using VSEARCH v2.30.0 (Rognes et al. 2016). VSEARCH implements the UCHIME2 algorithm (Edgar 2016) an update of the popular UCHIME chimera detection algorithm with new modes optimized for high-resolution biological sequence reconstruction ("denoising") for chimera removal.

We compared sequences from our specimens to previously published mtCOI sequences from Palearctic *Bootanomyia* associated with oak galls (Stone et al. 2012). These included *B. stigmatizans* Fabricus and two distinct species previously identified within the *B. dorsalis* morphology (Nicholls et al. 2010, 2018). We included a *Bortesia* sp. Pagliano & Scaramozzino as an outgroup, as well as five Palearctic *Megastigmus* Dalman species. We aligned all sequences using the MUSCLE algorithm (Edgar 2004) in Uniprot's Ugene v50 (Okonechnikov et al. 2012). We used IQ-Tree v2.0.7 (Minh et al. 2020) and ModelFinder (Kalyaanamoorthy et al. 2017) using 5,000 ultra-fast bootstraps and an approximate likelihood ratio test (aLRT; Guindon et al. 2010) to perform maximum likelihood analysis and infer a phylogeny for visualizing the relationship between sequences. The unpruned tree (Suppl. material 1: fig. S1) contained 19 sequences from the Pacific coast. Several were identical sequences from the same collection sites, and we pruned some of these sequences from the tree for ease of viewing. Gene tree pruning/stylizing was completed using the "ape" package in R v4.3.3 (Paradis and Schliep 2019; R Core Team 2024). Sequence similarity comparisons, in the form of percent identity, were made using the "seqidentity" function from the "bio3d" package in R v4.3.3 (Grant et al. 2006). Data for all samples used in molecular work can be found in Suppl. material 1: table S2.

Data accessibility

mtCOI sequences are available on GenBank (accessions PV174173–PV174193). Script for demultiplexing sequence data, developing distance matrices, and stylizing the gene tree is available on Zenodo (https://zenodo.org/records/15555447).

Results

Collections and morphological identification

We reared *Bootanomyia* wasps from six different oak gall wasp morphotypes collected on three oak tree species (Table 1). All 2,431 Pacific coast wasps were associated with galls on *Quercus garryana* (the only oak sampled). Five NY wasps emerged

Table 1. North American oak gall species from which *B. dorsalis* parasites were reared, the location from which they were collected, the generation of the gall, the tree species from which they were collected, and the taxonomic section to which the host tree belongs.

Bootanomyia species	Gall Wasp Species	Location	Gall Generation	Host Tree	Tree Section	# Reared	# Sequenced
B. dorsalis sp. 1	*Neuroterus quercusbatatus* (Fitch)	NY	unisexual	*Quercus bicolor*	Quercus	4	3
B. dorsalis sp. 1	*Acraspis erinacei* (Beutenmüller)	NY	unisexual	*Quercus alba*	Quercus	1	1
B. dorsalis sp. 2	*Andricus opertus* (Weld)	BC	bisexual	*Quercus garryana*	Quercus	25	4
B. dorsalis sp. 2	*Neuroterus saltatorius* Edwards	BC, OR	unisexual	*Quercus garryana*	Quercus	52	3
B. dorsalis sp. 2	*Neuroterus washingtonensis* Beutenmüller	WA, BC	bisexual	*Quercus garryana*	Quercus	2307	12
B. dorsalis sp. 2	*Besbicus mirabilis* (Kinsey)	WA	unisexual	*Quercus garryana*	Quercus	1	0

from galls on *Quercus bicolor* Willd. and *Quercus alba* L., both in the oak section Quercus. Pacific coast samples matched the description of *Bootanomyia dorsalis*, though we noticed variation in the extent of wing infumation, ranging from the typical expected from this species (extending from the stigma to the middle of the wing) to no or hardly any infumation (Fig. 1A, B). The Brooklyn, New York collection (2576-410-E14) also keyed to *B. dorsalis*, except the wing infumation, as in some Pacific coast samples, was not as strong as expected for that species. Doğanlar (2011) suggests that *B. dorsalis* has three body color "forms": "typical", "pale", and "dark". The specimens from the Pacific coast better match the pale form whereas those from NY are closer to the dark form (Fig. 1C, D).

Genetic identification

The mtCOI gene tree sorts the North American *B. dorsalis* into two clades, each also containing sequences from Palearctic *B. dorsalis* (Fig. 2). Sequences from NY specimens were 98.4–99.4% similar to sequences from *B. dorsalis* sp. 1 from Portugal, Iran, and Italy (Nicholls et al. 2018). Sequences from Pacific coast specimens were 97.9–98.1% similar to sequences from *B. dorsalis* sp. 2 from Spain, Hungary, and Iran. Sequences in the *B. dorsalis* sp. 1 and sp. 2 clades are 5.3–6.6% different from each other. Sequences from *B. dorsalis* sp. 1 collected from New York are 98.7%–100% similar to each other. All wasps from the Pacific coast were 100% identical at the mtCOI locus. Genetic sampling from the Pacific coast was more extensive, totaling 19 individuals from three different oak gall hosts from six sites in both the USA and Canada.

Discussion

These results suggest that two different species of oak gall-associated *Bootanomyia* wasps have been introduced from the Palearctic to the Nearctic. One introduction, to the Pacific coast of North America shares a single mtCOI haplotype closest to

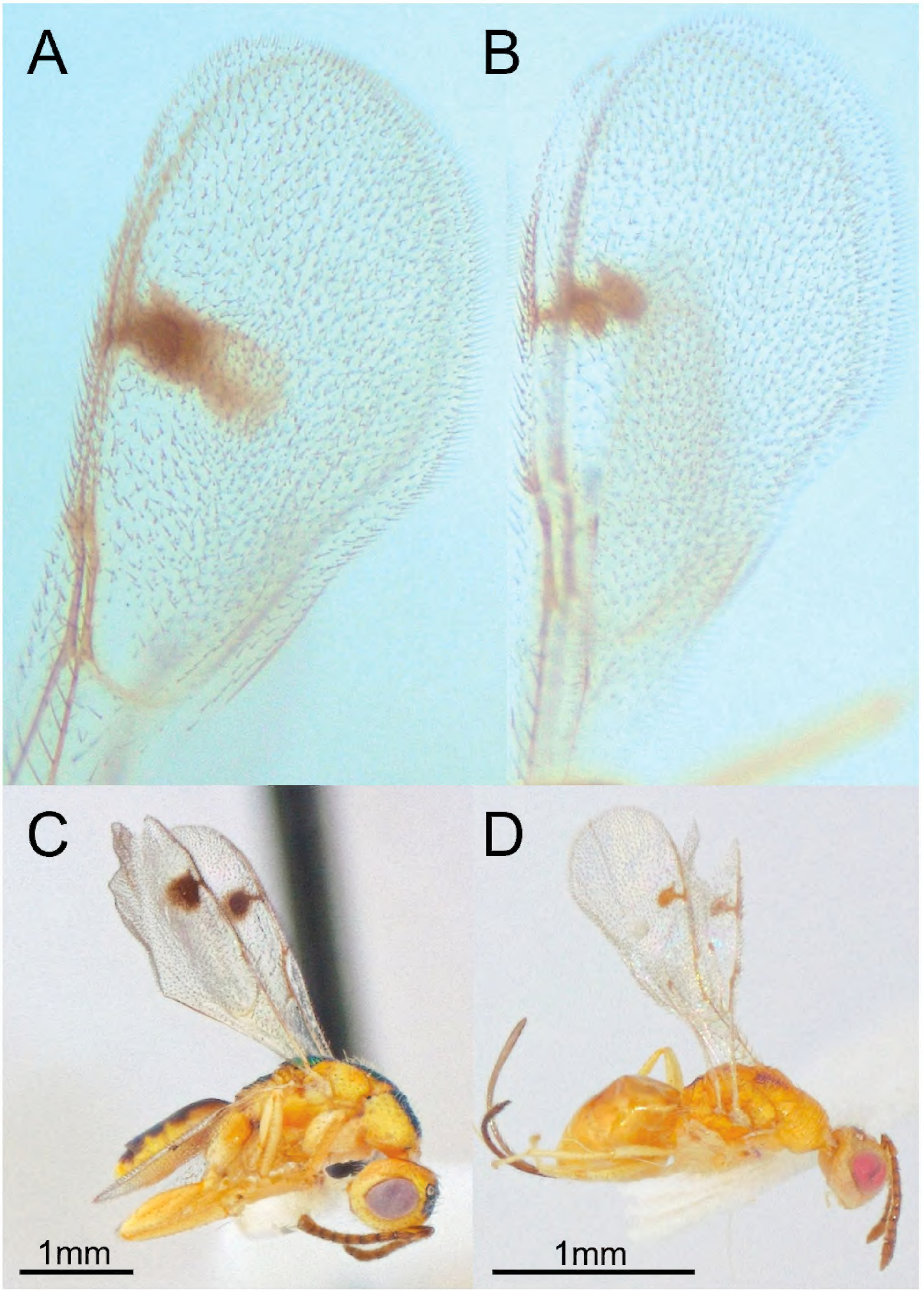

Figure 1. Two species of *Bootanomyia dorsalis* wasps introduced to North America from Europe **A, B** variation in the extent of wing infumation from a single collection of *B. dorsalis* sp. 2 from *Neuroterus washingtonensis* in Metchosin, BC **C** a male *B. dorsalis* sp. 1 collected from New York **D** a female *B. dorsalis* sp. 2 from the Pacific coast of North America. Body coloration of both **C** and **D** wasps are representative of their respective species regardless of sex.

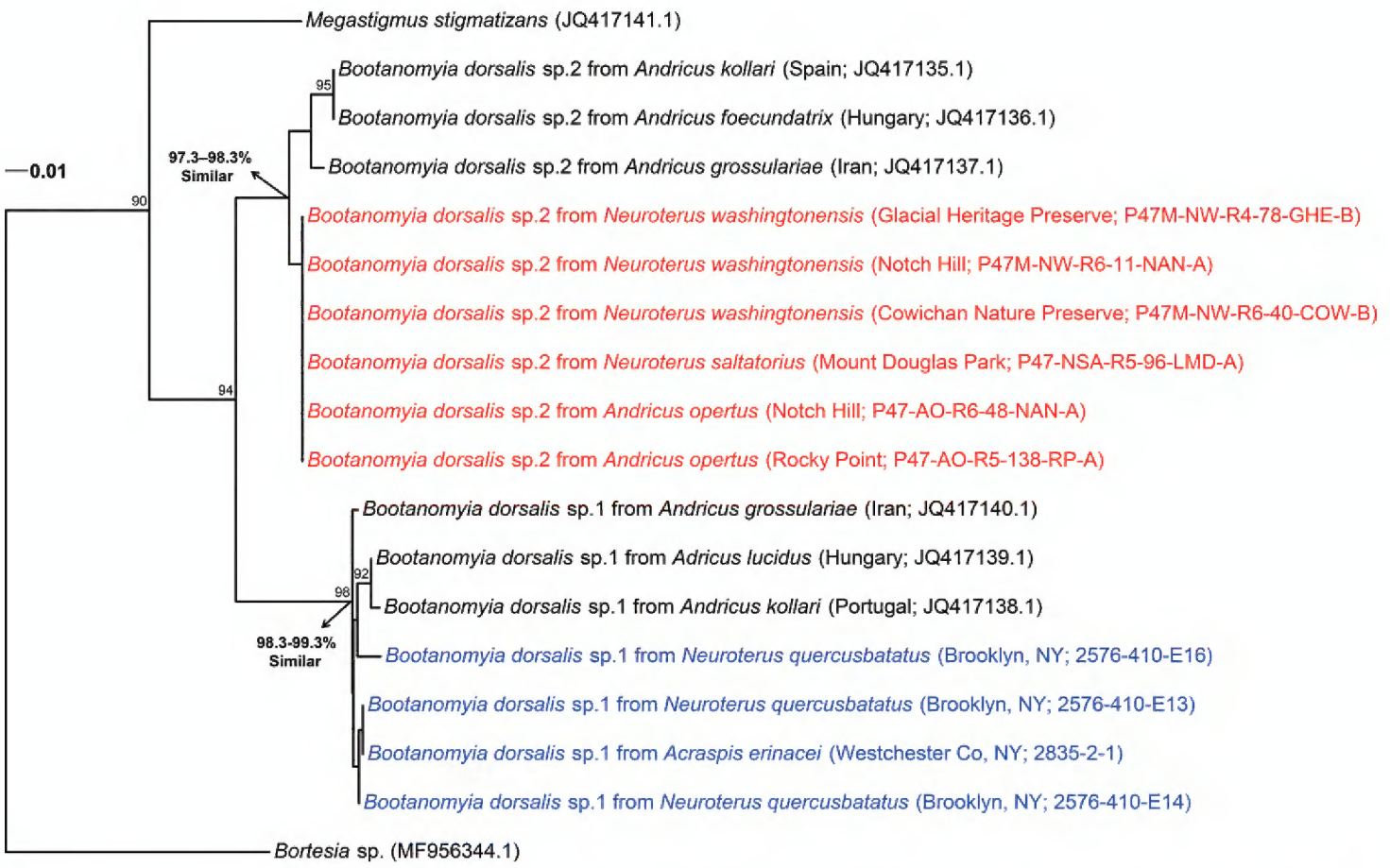
Megastigmus stigmatizans (JQ417141.1)
Bootanomyia dorsalis sp.2 from Andricus kollari (Spain; JQ417135.1)
Bootanomyia dorsalis sp.2 from Andricus foecundatrix (Hungary; JQ417136.1)
Bootanomyia dorsalis sp.2 from Andricus grossulariae (Iran; JQ417137.1)
Bootanomyia dorsalis sp.2 from Neuroterus washingtonensis (Glacial Heritage Preserve; P47M-NW-R4-78-GHE-B)
Bootanomyia dorsalis sp.2 from Neuroterus washingtonensis (Notch Hill; P47M-NW-R6-11-NAN-A)
Bootanomyia dorsalis sp.2 from Neuroterus washingtonensis (Cowichan Nature Preserve; P47M-NW-R6-40-COW-B)
Bootanomyia dorsalis sp.2 from Neuroterus saltatorius (Mount Douglas Park; P47-NSA-R5-96-LMD-A)
Bootanomyia dorsalis sp.2 from Andricus opertus (Notch Hill; P47-AO-R6-48-NAN-A)
Bootanomyia dorsalis sp.2 from Andricus opertus (Rocky Point; P47-AO-R5-138-RP-A)
Bootanomyia dorsalis sp.1 from Andricus grossulariae (Iran; JQ417140.1)
Bootanomyia dorsalis sp.1 from Adricus lucidus (Hungary; JQ417139.1)
Bootanomyia dorsalis sp.1 from Andricus kollari (Portugal; JQ417138.1)
Bootanomyia dorsalis sp.1 from Neuroterus quercusbatatus (Brooklyn, NY; 2576-410-E16)
Bootanomyia dorsalis sp.1 from Neuroterus quercusbatatus (Brooklyn, NY; 2576-410-E13)
Bootanomyia dorsalis sp.1 from Acraspis erinacei (Westchester Co, NY; 2835-2-1)
Bootanomyia dorsalis sp.1 from Neuroterus quercusbatatus (Brooklyn, NY; 2576-410-E14)
Bortesia sp. (MF956344.1)
0.01
97.3–98.3% Similar
98.3-99.3% Similar
95
90
94
92
98

Figure 2. Pruned and stylized mtCOI maximum likelihood phylogenetic tree of *Bootanomyia dorsalis* and close relatives. Ultra-fast bootstrap scores > 90 are shown next to their respective nodes. Tip labels highlighted in red were collected from the Pacific coast. Sequences from the same collection site and host gall have been removed for simplicity (see Suppl. material 1: fig. S1 for the complete tree). Sequences highlighted in blue were collected from samples in New York. All other sequences were acquired from previously published data. GenBank accession numbers for these sequences can be found in Suppl. material 1: table S2. Similarity scores are shown for clades where Nearctic and Palearctic sequences are both present. The score represents the range of genetic distance between Nearctic and Palearctic sequences.

those previously sequenced for *B. dorsalis* sp. 2. The lack of mtCOI genetic variation for the Pacific coast *B. dorsalis*, which are variable in the Palearctic (Nicholls et al. 2018), is a strong indicator of a population bottleneck, consistent with a founder effect upon introduction. The second introduction, discovered via two independent collections in New York, have similar mtCOI haplotypes to Palearctic *B. dorsalis* sp. 1. The New York introduction appears to be less bottlenecked, as evidenced by there being at least three distinct mtCOI haplotypes.

What are some possible pathways of introduction for *Bootanomyia* parasites of oak galls? *B. dorsalis* has only been recorded as a parasite of oak galls (Doğanlar 2011; Askew et al. 2013), so one possibility is that they were introduced along with Palearctic oak trees infested with galls. Nicholls et al. 2010 and 2018 record *B. dorsalis* emerging from 23 oak gall hosts on oak species including *Quercus robur* L. (sect. Quercus) and *Q. cerris* L. (sect. Cerris). Both trees have been introduced to North America and *Q. robur* has been widely planted since the 17th century and has been documented in the Canadian provinces of British Columbia, Ontario, and New Brunswick along with the U.S. states Ohio, Pennsylvania, New York,

Massachusetts, New Hampshire, and Maine (USDA Plants Database Plant Profile General 2024). *Q. cerris* has been documented on iNaturalist across the east coast, including one within 13 kilometers of a location where a *B. dorsalis* was collected. We also find one iNaturalist observation of *Neuroterus quercusbaccarum* (L.) (a Palearctic gall wasp host of *B. dorsalis*) in Papineauville, QC, Canada (approx. 525 kilometers from the NY discovery). *Neuroterus quercusbaccarum* is one of just two Palearctic oak gall hosts of *B. dorsalis* known to have been introduced to North America (Suppl. material 1: table S3). Alternatively, perhaps these wasps were introduced separately from their hosts or host trees. Adult females of other megastigmid species have a maximum longevity of 27 days (Bouaziz and Roques 2006), ample time for an inconspicuous insect to hitchhike in cargo or on a passenger plane across the Atlantic.

Are the introduced North American *Bootanomyia* parasites of cynipid oak galls also invasive? The ecological effects of parasitoid introductions are not well documented but could be substantial. The eupelmid wasp *Eupelmus messene* Walker may be the earliest documented parasitoid introduction (Burks 1979). Now suspected to be a complex of two or more species (Fusu, 2017), *E. messene* wasps attack and kill immature insects across hundreds of hosts from at least six orders in their introduced range (Gibson 1990). The braconid wasp *Cotesia glomerata* (L.), accidentally introduced to the Canary Islands, threatens the endemic butterfly *Pieris cheiranthi* (Hübner) (Lepidoptera: Pieridae; Lozan et al. 2008). Introduced parasitoids can also affect interactions other parasites have with their hosts. The introduction of *Heterospilus prosopidis* Viereck (Hymenoptera: Braconidae) as a secondary biological control agent for the azuki bean beetle, *Callosobruchus chinensis* (L.) (Coleoptera: Chrysomelidae), disrupts the efficacy of the native parasitoid *Anisopteromalus calandrae* (Howard) (Hymenoptera: Pteromalidae) (Tuda and Shimada 2005; Nakamichi et al. 2020).

It remains to be seen whether these two specific introductions of *Bootanomyia* wasps to North America present an existential threat to native gall wasps or other gall-associated insects. The Pacific coast introduction is already geographically widespread, and a bugguide.net report of *Bootanomyia* that appears to be *B. dorsalis* was recorded in Groton, MA on August 10th, 2021 (roughly 240 kilometers from the discovery in NY), suggesting the east coast introduction also has a wider distribution. Regardless of the extent of introduction, the primary relevance of documenting these introductions is in what they portend for the myriad other parasitoid communities that are not well-studied or regularly sampled. This finding of multiple introductions into the North American oak gall system – one of them apparently quite well-established across the Pacific coast – makes it reasonable to presume that the portcullis has been raised for parasitic wasp invasions, as it has been for so many other insects (Hokkanen and Lynch 2003). Efforts towards detection and identification of newly introduced species should consider investing in methods and resources that are inclusive of parasitic wasps.

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Supplementary material 1

Supplementary information

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